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Abstract: During sepsis, excessive activation of the complement system with generation of the anaphylatoxin C5a results in profound disturbances in crucial neutrophil functions. Moreover, because neutrophil activity is highly dependent on intracellular pH (pHi), we propose a direct mechanistic link between complement activation and neutrophil pHi In this article, we demonstrate that in vitro exposure of human neutrophils to C5a significantly increased pHi by selective activation of the sodium/hydrogen exchanger. Upstream signaling of C5a-mediated intracellular alkalinization was dependent on C5aR1, intracellular calcium, protein kinase C, and calmodulin, and downstream signaling regulated the release of antibacterial myeloperoxidase and lactoferrin. Notably, the pH shift caused by C5a increased the glucose uptake and activated glycolytic flux in neutrophils, resulting in a significant release of lactate. Furthermore, C5a induced acidification of the extracellular micromilieu. In experimental murine sepsis, pHi of blood neutrophils was analogously alkalinized, which could be normalized by C5aR1 inhibition. In the clinical setting of sepsis, neutrophils from patients with septic shock likewise exhibited a significantly increased pHi These data suggest a novel role for the anaphylatoxin C5a as a master switch of the delicate pHi balance in neutrophils resulting in profound inflammatory and metabolic changes that contribute to hyperlactatemia during sepsis.

DOI: https://doi.org/10.4049/jimmunol.1700393

The Journal of Immunology

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J Immunol 2017; 198:4846-4854; Prepublished online 10 May 2017;
doi: 10.4049/jimmunol.1700393
http://www.jimmunol.org/content/198/12/4846

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

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During sepsis, excessive activation of the complement system with generation of the anaphylatoxin C5a results in profound disturbances in crucial neutrophil functions. Moreover, because neutrophil activity is highly dependent on intracellular pH (pH_i), we propose a direct mechanistic link between complement activation and neutrophil pH_i. In this article, we demonstrate that in vitro exposure of human neutrophils to C5a significantly increased pH_i by selective activation of the sodium/hydrogen exchanger. Upstream signaling of C5a-mediated intracellular alkalinization was dependent on C5aR1, intracellular calcium, protein kinase C, and calmodulin, and downstream signaling regulated the release of antibacterial myeloperoxidase and lactoferrin. Notably, the pH shift caused by C5a increased the glucose uptake and activated glycolytic flux in neutrophils, resulting in a significant release of lactate. Furthermore, C5a induced acidification of the extracellular micromilieu. In experimental murine sepsis, pH_i of blood neutrophils was analogously alkalinized, which could be normalized by C5aR1 inhibition. In the clinical setting of sepsis, neutrophils from patients with septic shock likewise exhibited a significantly increased pH_i. These data suggest a novel role for the anaphylatoxin C5a as a master switch of the delicate pH_i balance in neutrophils resulting in profound inflammatory and metabolic changes that contribute to hyperlactatemia during sepsis. ***The Journal of Immunology*****, 2017, 198: 4846–4854.**

After severe tissue trauma or infections, patients are exposed to various danger- and pathogen-associated molecular patterns that trigger a robust activation of the complement and neutrophil defense systems, which are clinically manifested as systemic inflammatory response syndrome or sepsis (1, 2). The complement activation product C5a functions as a central molecule in the development of the overwhelming inflammatory response in sepsis (3, 4). Studies revealed that high levels of C5a and its interaction with C5aR1 resulted in overactivation of central neutrophil functions, including cytoskeletal reorganization, chemotaxis, phagocytosis, cytokine release, and respiratory burst (5, 6). Remarkably, all of these cellular functions were reported to be dependent on intracellular pH (pH_i) modulation (7–10). Moreover, C5a and cellular pH changes effectively control apoptosis, as well as drive tumor growth and invasion (11, 12). In these pathological and inflammatory conditions, the pH_i homeostasis of cells is substantially challenged due to a decreased extracellular pH microenvironment that results from enhanced anaerobic metabolism (13, 14). In addition, intracellular accumulation of detrimental acid equivalents generated by activation of the NADPH oxidase or the pentose phosphate pathway, for example, represents a potential risk for intracellular acidification of neutrophils (15, 16). Physiologically, neutrophils can minimize intracellular H^+ excess through several proton-extrusion systems, including the sodium/hydrogen exchanger 1 (NHE1), proton-translocating V-type adenosine triphosphatases (V-ATPases), and

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Received for publication March 15, 2017. Accepted for publication April 16, 2017.

This work was supported by the German Research Foundation by grants assigned to the Clinical Research Unit 200 (Grants HU823/3-1 and HU823/3-2), to the Collaborative Research Centre 1149 (A01) (all to M.S.H.-L.), and to the Institute for Systemic Inflammation Research (Research Program IRTG1911, to J.K.).

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Abbreviations used in this article: C5aR1A, C5aR1 antagonist; CLP, cecal ligation and puncture; DPBS, Dulbecco's PBS; HIF-1α, hypoxia-inducible factor-1α; MFI, mean fluorescence intensity; MPO, myeloperoxidase; mTOR, mammalian target of rapamycin; 2-NBDG, 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-6-deoxyglucose; NHE1, sodium/hydrogen exchanger 1; NHE1-Inhib, NHE1 inhibitor; PFK1, phosphofructokinase-1; pH_i, intracellular pH; PKC, protein kinase C; ROCK, Rho-associated protein kinase; V-ATPase, V-type adenosine triphosphatase.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1700393

NADPH oxidase–associated H^+ conductance channels (17–19). A well-adjusted regulation of these H^+ exporter systems is crucial to modulate neutrophil pH_i and especially to guarantee efficient microbicidal functions of neutrophils. In this context, it is established that cellular activation is further associated with an increased energy demand/consumption. Studies indicate that, in neutrophils, the energy required for chemotaxis, phagocytosis, and radical formation is primarily derived from glycolysis (20–22), whereas the few mitochondria mainly serve to control apoptosis (23, 24). Although an increased glycolytic activity has been proposed after stimulation with trypsin-digested C5, a direct linkage among complement activation, pH_i regulation, and metabolic activity has been not investigated.

In the present study, we propose that C5a, as a key mediator of innate immunity, tips the pH balance of neutrophils, exerting effects on fundamental immune-modulatory and metabolic functions, especially under systemic inflammatory conditions.

Materials and Methods

Reagents

Unless otherwise indicated, all reagents were purchased from Sigma-Aldrich.

Isolation of human neutrophils

Whole blood from healthy volunteers was drawn into syringes containing 10% anticoagulant citrate dextrose (Fresenius Kabi) within a prospective observational clinical study, as approved by the Independent Local Ethics Committee of the University of Ulm (approval number 16303). Neutrophils were isolated using Ficoll-Paque gradient centrifugation (Amersham Biosciences), followed by a dextran sedimentation step. After hypotonic lysis of residual erythrocytes, neutrophils were resuspended, as specified below, in Dulbecco's PBS (DPBS; Life Technologies) or HBSS (Life Technologies).

Neutrophil stimulation

Isolated human neutrophils (2×10^6 cells per milliliter, in DPBS) were incubated in the presence or absence of human rC5a (0.1–100 nM) or C3a (0.1–100 nM; both from Calbiochem) for 1–30 min at 37–C. For blockade of C5aR1, neutrophils were treated with increasing concentrations (0.01–10 μM) of the selective small peptide C5aR1 antagonist (C5aR1A), AcF [OPdChaWR] (25, 26), for 10 min at 37˚C.

Inhibition of proton-extrusion and intracellular signaling mechanisms

To investigate different mechanisms of proton extrusion, isolated human neutrophils were incubated at 37˚C with a hydrogen potassium ATPase inhibitor (omeprazol, 10 μM, 10 min), a V-ATPase inhibitor (bafilomycin A1, 0.1 μM, 5 min), an inhibitor of NADPH oxidase–associated H^+ channels (zinc chloride, 50 μM, 5 min), a sodium exchanger inhibitor (amiloride hydrochloride hydrate, 200 μM, 5 min) or an acylguanidine derivate [(4-cyanobenzo[b]thiophene-2-carbonyl)guanidine, methanesulfonate] (Calbiochem) as a specific NHE1 inhibitor (NHE1-Inhib; 5 μM, 10 min). For assessment of underlying signaling mechanisms, neutrophils were exposed to BAPTA-AM (10 μM, 60 min), PD98059 (50 μM, 60 min), staurosporine (0.7 μM, 30 min), Y-27632 (10 μM, 30 min) (BioSource), thapsigargin (2 μM, 30 min), SKF-96365 (12.5 and 50 μM, 30 min), EDTA (10 mM, 10 min), or the calmodulin antagonist W-7 (40 μM, 30 min) (Biomol).

Sodium-free medium

To compare cellular responses in Na^+-containing and Na^+-free medium, neutrophils were resuspended in buffers containing 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, 10 mM glucose, and either 140 mM NaCl (pH 7.3) or equiosmolar amounts of *N*-methyl-D-glucamine (pH 7.3).

Neutrophil pH_i

For measurement of pH_i, neutrophils were incubated with 1 μM carboxy-seminaphthorhodafluor-1 acetoxymethyl ester acetate (Life Technologies) for 20 min at 37˚C. After washing, cells were resuspended in DPBS. Flow cytometric measurement was performed using a FACSCanto II cytometer (BD Biosciences). The ratio of emitted mean fluorescence intensities (MFIs) at 585 and 682 nm, after excitation at 488 nm, was determined. MFI ratios were converted to pH units using the nigericin/high K^+ method of calibration, as previously described (27).

Extracellular pH

Isolated human neutrophils (5×10^6 cells per milliliter) were resuspended in HBSS without Ca^{2+}/Mg^{2+} + 0.1% glucose. Cells were pretreated with C5aR1A (10 μM, for 10 min) before stimulation with human rC5a (10 nM, for 15 min) at 37˚C in a total volume of 500 μl. The extracellular pH was determined in the cell supernatant (800 × *g*, 5 min, 4˚C) using a clinical blood gas analyzer (Radiometer ABL700 Series; Radiometer, Copenhagen, Denmark).

Myeloperoxidase and lactoferrin assays

For assessment of myeloperoxidase (MPO) activity, neutrophil supernatant or standard human MPO (Merck) was incubated with tetramethylbenzidine (100 μg/ml) and H_2O_2 (0.0016%) at 37˚C. After 5 min, the reaction was terminated by the addition of 2 M H_2SO_4, and the absorbance was determined using a spectrophotometric reader at 450 nm (CytoFluor Series 4000; Perspective Biosystems). Concentrations of lactoferrin in neutrophil supernatants were measured using a commercially available ELISA kit, specific for human lactoferrin, according to the manufacturer's instructions (Assaypro).

Lactate production

The glycolytic activity of neutrophils was determined by measuring the extracellular lactate concentration. Isolated human neutrophils (5×10^6 cells per milliliter, in HBSS without Ca^{2+}/Mg^{2+} + 0.1% glucose) were incubated in the presence or absence of human rC5a (1–100 nM) for 15 min at 37˚C. For C5aR1 blockade, cells were pretreated with C5aR1A (10 μM) before C5a stimulation (10 nM). The $L(^+)$-lactate concentration was determined in the cell supernatant (800 × *g*, 5 min, 4˚C) using the commercially available lactate assay kit II.

Glucose-uptake assays

Glucose uptake was analyzed using the fluorescent glucose analog 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-6-deoxyglucose (2-NBDG; Molecular Probes). Neutrophils were equilibrated in DPBS and pretreated with C5aR1A (10 μM), NHE1-Inhib (50 μM), or DPBS (control) for 10 min at 37˚C. 2-NBDG (15 μM) was added, and basal 2-NBDG uptake was determined as FITC MFI by flow cytometry. Neutrophils were then stimulated using C5a (10 nM), and 2-NBDG uptake was measured after 1, 5, and 10 min. The C5a-induced glucose uptake was calculated as the fold increase between the C5a-stimulated 2-NBDG uptake and the basal 2-NBDG uptake for each sample.

Determination of glycolytic flux and relative activity of the pentose-phosphate shunt

To determine the origin of lactate in the neutrophil supernatant fluid, gas chromatography/mass spectrometry was performed. Isolated human neutrophils (5×10^6 cells per milliliter) were incubated with C5a (15 min) in the presence or absence of the NHE1-Inhib (50 μM) in 50% 1,2-[^{13}C] glucose–enriched HBSS medium. After incubation, a 100-μl sample of the incubation medium before and after incubation was spiked with 50 μg 1,2,3-^{13}C–labeled lactate. The dried and spiked samples were derivatized with 25 μl of *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (abcr) to form the *tert*-butyldimethylsilyl derivative of lactate as the derivative. The derivatized samples were injected into an Agilent 6980 gas chromatograph/5975 mass spectrometer system, and the mass distribution of the molecular ion was assessed in the range *m/z* 261–265 by integration of the corresponding mass traces. The mass distribution was converted to the mole fractions of lactate molecules carrying 0, 1, 2, or 3 labeled carbons (M0, M1, M2, M3) using the approach of Vogt et al. (28). The (M0+M1+M2)/M3 ratio was taken as the ratio of the lactate amount produced by granulocytes over the amount of the 1,2,3-[^{13}C] lactate spike, and the difference between pre- and postincubation values was used to assess the amount of lactate accumulated during incubation. The M2/M1 ratio was used to assess the fractional contribution of the pentose-phosphate shunt to the lactate accumulation using the formulas of Lee et al. (29).

Animals

For experimental sepsis, male C57BL/6 mice (weighing 25–30 g; purchased from the Jackson Laboratory) were used. All animals had unrestricted access to food and water. The in vitro stimulation experiments were conducted on neutrophils from homozygous C5aR1 ($C5aR1^{-/-}$) or

C5aR2 ($C5aR2^{-/-}$) gene–knockout mice on a C57BL/6 background and strain-matched wild-type animals (5–6 mo old, weighing 25–35 g). The knockout mice were generated by Dr. C. Gerard (Boston Children's Hospital, Boston, MA).

Experimental sepsis by cecal ligation and puncture

The study was approved by the University of Ulm Committee on the Use and Care of Animals (approval number 988). Mice from the same litters were randomly assigned to each experimental group. Experimental sepsis was induced by cecal ligation and puncture (CLP), as previously described (30). In brief, after abdominal midline incision, 50% of the cecum was ligated below the ileocecal valve (midgrade CLP). The cecum was then punctured by a single through-and-through perforation (21-gauge needle), and a small portion of feces was extruded. Mice received buprenorphine (0.01 mg/kg body weight) s.c. every 8 h after surgery. For blockade of C5aR1, mice received a single i.v. injection of C5aR1A AcF[OPdChaWR] (1 μg/g body weight) in 300 μl of sterile DPBS immediately after the CLP procedure in a nonblinded manner; control animals were injected with a scrambled control peptide at the same concentration. Sham-operated mice received sterile DPBS (300 μl) after undergoing identical procedures as CLP mice, with the exception of ligation and puncture of the cecum. Whole blood was collected 24 h after surgery into EDTA-containing syringes, and neutrophil pH_i was determined by flow cytometry. No animal or extracted sample was excluded from any of the analyses, with the exception of nonsurviving septic animals.

Clinical sepsis

Ten patients who fulfilled the clinical criteria for severe sepsis or septic shock (based on the guidelines of the American College of Chest Physicians/Society of Critical Care Consensus Conference Committee) were analyzed within a prospective observational monocenter study (NCT00736723). Ten sex- and age-matched healthy volunteers served as a control group. Blood was drawn from patients in the early phase of septic shock (within 6 h), and blood samples from patients and volunteers were analyzed for neutrophil pH_i by flow cytometry. Written informed consent was obtained for all study participants. The study was performed with the permission of the Independent Local Ethics Committee of the University of Ulm (approval number 11407).

Statistical analysis

Results are presented as mean ± SEM; *n* values indicate the numbers of different donors used. Donors were used only once per experiment to account for donor-to-donor variability. Statistical analysis was performed using SigmaStat 3.5 (Systat Software). Data sets were analyzed using one-way ANOVA, followed by the Student–Newman–Keuls test as a post hoc test for multiple comparisons. In the case of nonparametric distribution, Kruskal–Wallis ANOVA on ranks, followed by the Dunn method, was performed. For analysis of differences between two normally distributed groups, a two-tailed *t* test was used. The p values <0.05 were considered statistically significant.

Results

C5a-induced intracellular alkalinization of neutrophils

To assess the effects of the anaphylatoxin C5a on pH_i regulation of neutrophils, complement activation was first simulated in vitro. Isolated human neutrophils were incubated with DPBS alone (control) or with 10 nM human rC5a for 1–30 min at 37°C, and its potential impact on neutrophil pH_i was determined by flow cytometry. At concentrations found in plasma during sepsis (31–33), C5a induced a rapid and transient elevation in pH_i, reaching its maximum 5 min after stimulation (Fig. 1A). This alkalinization was dose dependent and reached a plateau at C5a concentrations $>$ 2.5 nM, whereas human C3a failed to significantly alter pH_i (Fig. 1B). To characterize the specificity of the C5a effect and to determine the role of C5aR1 in the observed pH_i increase, neutrophils were treated with increasing concentrations (0.01–10 μM) of the selective small peptide C5aR1A AcF[OPdChaWR] (25) prior to C5a stimulation; control cells were incubated with DPBS only. As shown in Fig. 1C, C5aR1A reduced the C5a-induced alkalinization in a dose-dependent manner. At C5aR1A concentrations $\geq$ 1 μM, the C5a effect was completely abolished, indicating that the C5a-induced increase in pH_i was dependent on upstream C5aR1 signaling.

C5a-induced pH_i increase is mediated by NHE1

To define the proton-expelling mechanisms involved in the C5a-triggered pH_i response in an initial screening, neutrophils were treated with inhibitors of the main pH_i regulatory pumps and channels prior to C5a stimulation. Blockade of hydrogen potassium–ATPase, V-ATPases, or NADPH oxidase–associated H^+ channels all failed to affect the C5a-induced pH_i increase (Fig. 2A). In striking contrast, blockade of sodium channels by amiloride (Fig. 2A) completely abolished the C5a effect. To further characterize the involved transporter, we proceeded to specifically block NHE1 using a highly selective NHE1-Inhib (15), (4-cyanobenzo[b] thiophene-2-carbonyl)guanidine, methanesulfonate (Fig. 2B). Moreover, replacement of extracellular Na^+ (by equiosmolar amounts of *N*-methyl-D-glucamine) as a driving force for NHE1 led to a marked decrease in the resting pH_i of unstimulated cells, suggesting a relevant role for NHE1 in the regulation of pH_i under basal conditions. Subsequent exposure of neutrophils to C5a prevented the C5a-induced pH_i increase (Fig. 2C), demonstrating that the complement-mediated pH_i regulation in neutrophils is highly dependent on NHE1 activity. In this regard, isolated neutrophils from $C5aR1^{-/-}$ mice were unable to increase their pH_i in response to C5a stimulation, which was contrary to the significant pH_i increase observed for neutrophils from wild-type or $C5aR2^{-/-}$ mice (Fig. 2D), ruling out a substantial contribution of C5aR2 to the observed pH shift.

C5a-induced activation of NHE1 is dependent on intracellular Ca^{2+}/calmodulin and protein kinase C

To assess the underlying NHE1 activation pathway, human neutrophils were treated with a series of signaling inhibitors before C5a stimulation (Table I). C5a-mediated pH_i changes were not significantly affected by PD98059 (50 μM for 60 min) as selective inhibitor of MEK-1 or Y-27632 (10 μM for 30 min) as inhibitor of the Rho-associated protein kinase (ROCK). In contrast, the intracellular Ca^{2+} chelator BAPTA-AM, as well as the protein kinase C (PKC) inhibitor staurosporine, significantly reduced the C5a-induced alkalinization. To further characterize the Ca^{2+}-dependent regulation of neutrophil pH_i, we used several inhibitors for blockade of intra- or extracellular Ca^{2+}. Thapsigargin, which depletes intracellular Ca^{2+} stores in neutrophils by inhibiting the reuptake of Ca^{2+} into the endoplasmic reticulum through Ca^{2+}-dependent ATPases (15, 34), significantly reduced the C5a-induced alkalinization of neutrophils. In contrast to thapsigargin, SKF-96365 selectively blocks receptor-mediated Ca^{2+} entry from the extracellular medium while sparing internal Ca^{2+} release at concentrations that cause ~50% inhibition of the Ca^{2+} influx (IC_{50} 11.2 ± 1.4 μM); at higher concentrations, additional effects on internal Ca^{2+} release could be demonstrated (35). Preincubation of neutrophils with 12.5 μM SKF-96365 did not affect pH_i regulation by C5a, whereas the C5a effect was significantly decreased in the presence of 50 μM SKF-96365. The role of extracellular Ca^{2+} was further assessed by the addition of 10 mM EDTA to the stimulation medium as a chelator of external Ca^{2+}. However, no reduction in the C5a-induced decrease in pH_i could be detected after depletion of extracellular Ca^{2+}. To discover a potential link between the Ca^{2+} dependency of the C5a response and the direct regulation of NHE1, involvement of the Ca^{2+}-binding protein calmodulin was determined. The calmodulin antagonist W-7 blocked the C5a-mediated pH shift (Table I), revealing a crucial contribution of Ca^{2+}/calmodulin in the activation cascade of NHE1 induced by C5a.

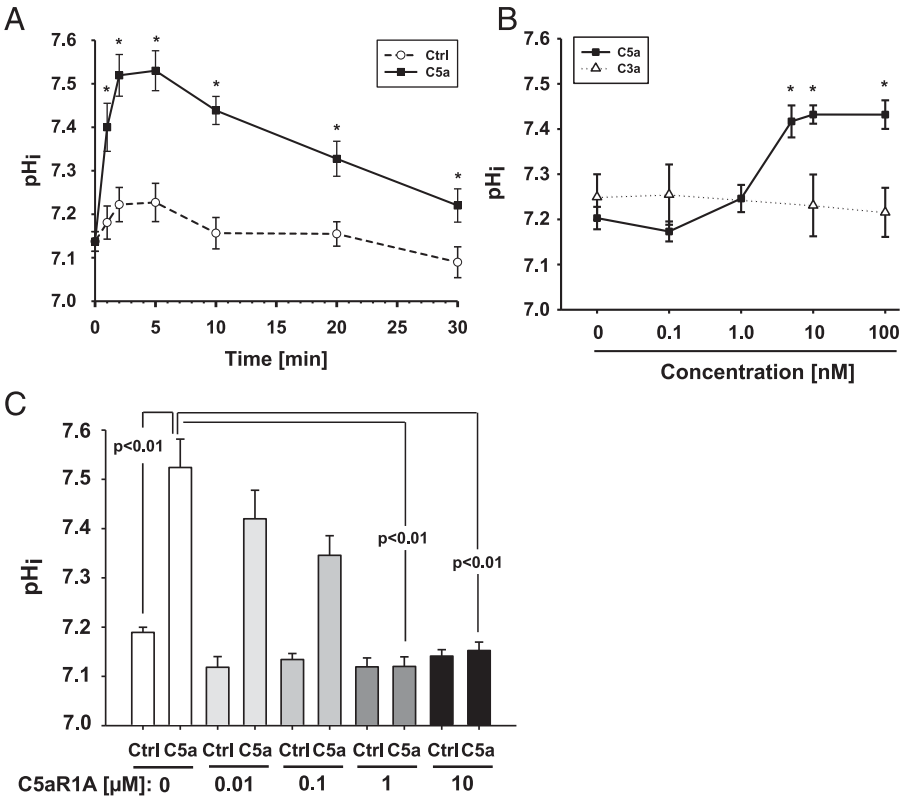

FIGURE 1. In vitro effects of C5a on pH_i of isolated neutrophils, as determined by flow cytometry. (**A**) Time course of human neutrophil pH_i after incubation with 10 nM human C5a or DPBS (control) for 1–30 min ($n = 7$). (**B**) Dose response of the pH_i change in neutrophils stimulated with 0.1–100 nM C5a or C3a for 5 min ($n = 7$). (**C**) Inhibitory effects of the selective C5aR1A AcF[OPd-ChaWR] (pretreatment for 10 min) on C5a-induced pH_i changes ($n = 5$). *$p < 0.05$, C5a versus control (A) or versus 0 nM C5a (B). Ctrl, control.

NHE1 blockade attenuates neutrophil degranulation

With regard to the functional effects downstream of the NHE1-mediated pH_i increase, we analyzed neutrophil degranulation as a hallmark of endogenous antibacterial defense. Short-term exposure of neutrophils to C5a already enhanced the release of azurophilic granules (MPO) and specific granules (lactoferrin), both of which were significantly decreased after NHE1 inhibition (Fig. 3). Thus, C5a-induced degranulation in neutrophils might be regulated by a pH_i-dependent mechanism.

C5a-induced activation of glycolytic flux is pH dependent

Because cellular activation, in general, is associated with increased energy demand/consumption, we determined the uptake of the fluorescent glucose analog 2-NBDG immediately after C5a stimulation. As depicted in Fig. 4A, C5a significantly increased the glucose uptake in neutrophils, which could be completely abolished by pretreatment with NHE1-Inhib or C5aR1A. Furthermore, in C5aR1-knockout mice, neutrophils were incapable of increasing the glucose uptake after stimulation with murine C5a (Fig. 4B).

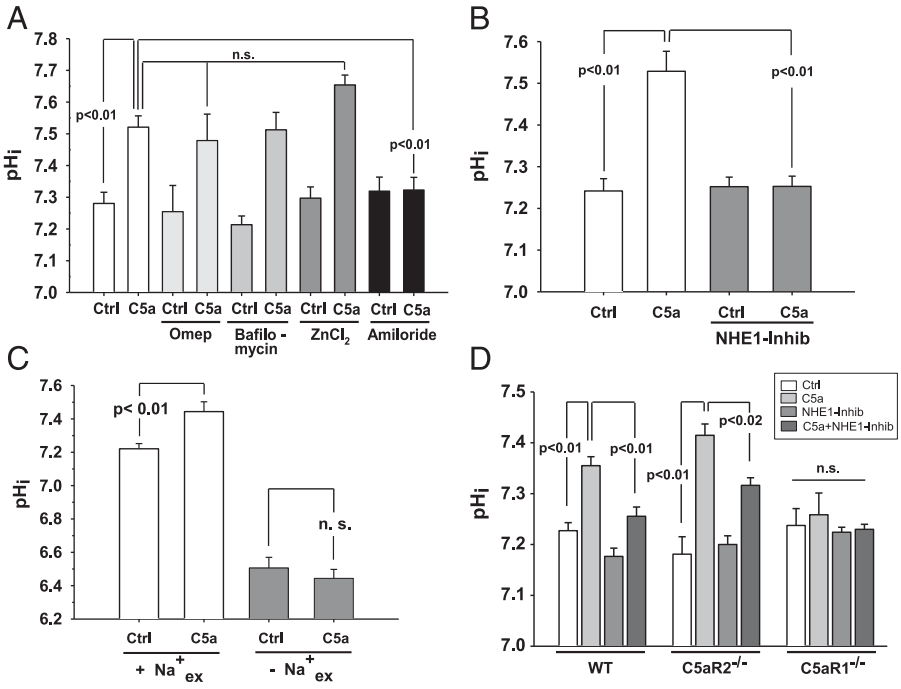

FIGURE 2. Contribution of putative mechanisms of proton extrusion to the C5a-induced pH_i change. Human neutrophils were exposed to 10 nM C5a for 5 min after blockade of hydrogen potassium ATPases (by 10 μM omeprazole [Omep]), V-ATPases (by 0.1 μM bafilomycin), NADPH oxidase–associated H^+ channels (by 50 μM $ZnCl_2$), and NHE1 (by 200 μM amiloride) (all $n = 6$) (**A**) or by 5 μM NHE1-Inhib ($n = 6$) (**B**) or by depletion of extracellular sodium (Na^+_{ex}) ($n = 7$) (**C**). (**D**) Effect of mouse rC5a on isolated neutrophils from wild-type (WT), C5aR2-knockout ($C5aR2^{-/-}$), and C5aR1-knockout ($C5aR1^{-/-}$) mice with/without pretreatment with NHE1-Inhib (5 μM, 10 min) ($n = 3$). Ctrl, control; n.s., not significant.

Table I. Effect of extra- and intracellular signaling modulators on the C5a-induced pH_i change in neutrophils

Modulator (Concentration, Preincubation Time)	C5a-Induced pH_i Changes: Versus Control (ΔpH Units)	C5a-Induced pH_i Changes: Versus Modulator (ΔpH Units)	Inhibition (%)	*p* Value[a]	Inhibition of
PD98059 (50 μM, 60 min)	0.209 ± 0.03	0.162 ± 0.03	22.5	0.28	MEK
Y-27632 (10 μM, 30 min)	0.227 ± 0.03	0.224 ± 0.05	1.3	0.94	ROCK
BAPTA-AM (10 μM, 60 min)	0.208 ± 0.03	0.054 ± 0.03	73.7	**0.009**	Intracellular Ca^{2+}
Staurosporine (0.7 μM, 30 min)	0.235 ± 0.03	0.002 ± 0.01	99.2	**<0.001**	PKC
Thapsigargin (2 μM, 30 min)	0.247 ± 0.02	0.057 ± 0.01	76.9	**<0.001**	Reuptake of Ca^{2+} into the ER
SKF-96365 (12 μM, 30 min)	0.235 ± 0.03	0.232 ± 0.04	1.3	0.95	Receptor-mediated uptake of $[Ca^{2+}]_{ex}$
SKF-96365 (50 μM, 30 min)	0.235 ± 0.03	0.075 ± 0.04	68.1	**0.006**	Ca^{2+} release from internal stores
EDTA (10 mM, 10 min)	0.243 ± 0.02	0.260 ± 0.06	0	1	$[Ca^{2+}]_{ex}$
W-7 (40 μM, 30 min)	0.221 ± 0.03	0.064 ± 0.04	71.0	**0.01**	Calmodulin

Freshly isolated human neutrophils were incubated in the absence (control) or presence of the indicated modulators before C5a stimulation (10 nM, 5 min; $n = 5–8$). At low concentrations (12 μM), SKF-96365 selectively blocks receptor-mediated Ca^{2+} entry, whereas at higher concentrations (50 μM), internal Ca^{2+} release is blocked.

[a] *p* values < 0.05 (bold) indicate a significant inhibitory effect of the modulator on the C5a-mediated pH_i change compared with control.

$[Ca^{2+}]_{ex}$, extracellular calcium; ER, endoplasmic reticulum.

To investigate whether the increased glucose uptake was associated with an increased glycolysis rate, we determined lactate generation by neutrophils as an indicator for glycolytic activity. As depicted in Fig. 4C, C5a resulted in a significant concentration-dependent increase in extracellular lactate generation. Gas chromatography/mass spectrometry analysis showed that the C5a-induced lactate completely originated from aerobic glycolysis compared with the pentose phosphate pathway (Fig. 4D). That the C5a-induced activation of glycolytic flux was sensitive to pH_i changes could be demonstrated by blocking this pathway with NHE1-Inhib (Fig. 4E), which significantly reduced the lactate generation due to C5a incubation. These results illustrate a novel mechanistic link between the complement system and immune-metabolic adaptation.

Sepsis-induced increase in pH_i in neutrophils is C5a dependent

Because acidosis is a frequent finding in patients with severe sepsis, we investigated whether C5a stimulation of neutrophils results in acidification of the extracellular micromilieu. Indeed, in vitro exposure of isolated neutrophils to C5a led to a significant decrease in pH of the external buffer within 15 min that could be normalized with the specific C5aR1A (Fig. 5A).

To extrapolate the pH_i findings into the complex in vivo setting of sepsis, we determined the pH_i of blood neutrophils from mice 24 h after CLP-induced sepsis or sham intervention. During fully established sepsis, pH_i of neutrophils was significantly increased compared with sham animals (Fig. 5B). Administration of C5aR1A (1 μg/g body weight) immediately after sepsis induction normalized the neutrophil pH_i, whereas a control peptide failed to do so.

In a clinical study, patients suffering from severe sepsis or septic shock caused by various underlying diseases all exhibited a significantly elevated neutrophil pH_i compared with age- and sex-matched healthy volunteers (Fig. 5C). Remarkably, the extent of the pH_i shift during sepsis was similar to the pH_i changes observed in septic animals and after in vitro exposure of neutrophils to C5a. These results indicate that, during sepsis, high levels of C5a lead to an increase in the neutrophil pH_i that is associated with functional and metabolic activation (Fig. 6).

Discussion

During inflammatory conditions, complement activation triggers the activation of crucial neutrophil functions. The modulation of neutrophil functionality is further highly dependent on pH_i regulation. In the current study, we demonstrate a direct mechanistic linkage between the complement system and the tightly regulated pH_i homeostasis of neutrophils that is decisive for functional and metabolic activation (Fig. 6). Interaction of C5a with the G protein–coupled C5aR1 was found to increase neutrophil pH_i by stimulation of NHE1, which resulted in increased degranulation and activation of glycolytic flux in neutrophils.

For the activation of NHE1, an intracellular proton load has been considered the driving force. However, NHE1 activity can also be regulated by different hormones, growth factors, and inflammatory mediators that are supposed to induce modifications (phosphorylation, binding) of the C-terminal cytoplasmic domain of the exchanger and, thus, alter the affinity for intracellular protons (15, 18). In most cell types, G protein–coupled regulation of NHE1 has been reported to be unresponsive to changes in cAMP; rather, it depends on Ca^{2+}/PKC, Ca^{2+}/calmodulin, or RhoA/ROCK signaling (15). In this regard, a high-affinity calmodulin binding site was described to regulate NHE1 activity in response to the Ca^{2+}-triggered signaling pathway (36, 37). Furthermore, tyrosine

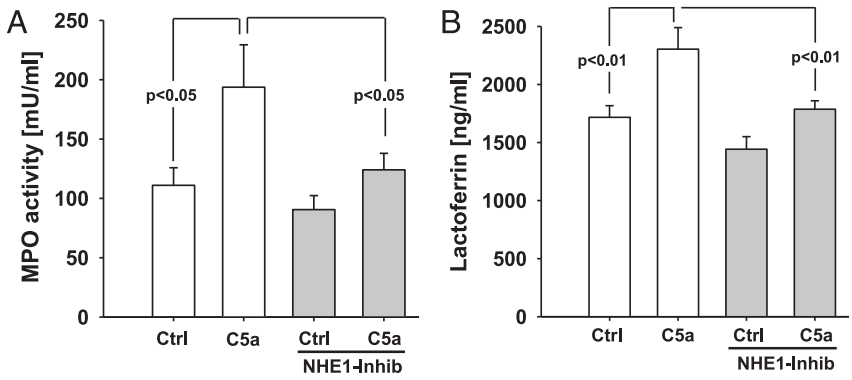

FIGURE 3. C5a-induced pH_i change affects neutrophil functions. MPO activity ($n = 15$) (**A**) and lactoferrin ($n = 9$) concentrations (**B**) were determined in supernatants of freshly isolated human neutrophils after exposure to NHE1-Inhib (5 μM), followed by stimulation with C5a (10 nM for 5 min). Ctrl, control.

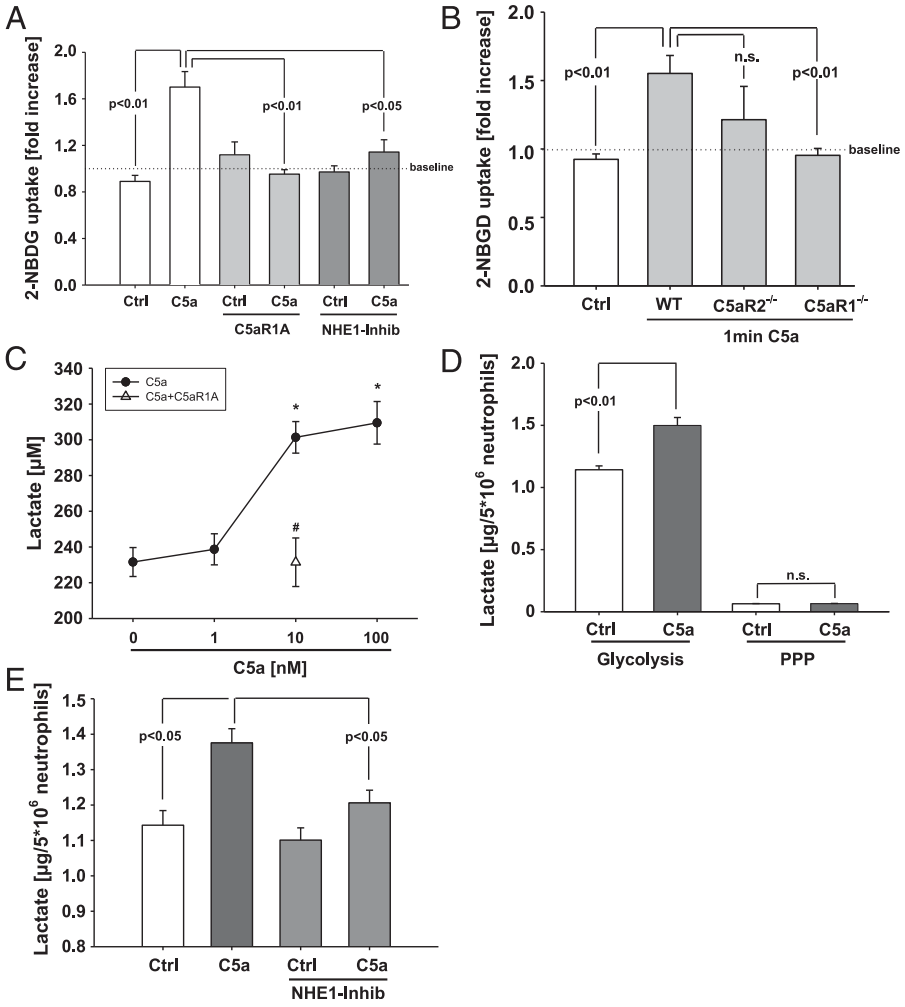

FIGURE 4. Effect of the C5a-induced pH_i change on human neutrophil metabolic activity. (**A**) Glucose uptake of neutrophils using the fluorescent glucose analog 2-NBDG. Neutrophils were treated with C5aR1A (10 μM), NHE1-Inhib (50 μM), or DPBS (Ctrl) for 10 min before C5a stimulation (10 nM, for 1 min) (n = 4–6). (**B**) 2-NBDG uptake of neutrophils from wild-type (WT), C5aR2-knockout ($C5aR2^{-/-}$), and C5aR1-knockout ($C5aR1^{-/-}$) mice 1 min after stimulation with murine C5a (10 nM) or DPBS (Ctrl) (n = 3–5). C5a-induced glucose uptake was calculated as the fold increase between the C5a-stimulated and the basal 2-NBDG uptake (before stimulation) for each sample (a + b). (**C**) Lactate concentration in the supernatant of neutrophils 15 min after incubation with increasing concentrations of C5a (n = 7); at 10 nM C5a, an additional set of neutrophils (n = 4) was treated with C5aR1A (10 μM) before C5a stimulation (n = 4). (**D**) Discriminative analysis of lactate generation in the neutrophil supernatant (5×10^6 cells) originated from glycolysis and the pentose phosphate pathway (PPP) after 15 min of C5a exposure, as assessed by gas chromatography/mass spectrometry (n = 10). (**E**) Amount of glycolysis-derived lactate in the neutrophil supernatant after pretreatment of 5×10^6 cells with NHE1-Inhib (50 μM), followed by C5a stimulation (15 min) (n = 8). $^*p < 0.05$ versus control, $^{\#}p < 0.05$ versus 10 nM C5a. Ctrl, control; n.s., not significant.

kinase receptors have been shown to increase NHE1 activity via cascades including MEK1/2 and MAPK. Results from the current study clearly demonstrate that C5a stimulation leads to Ca^{2+} release from internal stores, which subsequently induced direct activation of NHE1 via interaction of PKC and calmodulin. In contrast, an indirect NHE1-activation mechanism has been

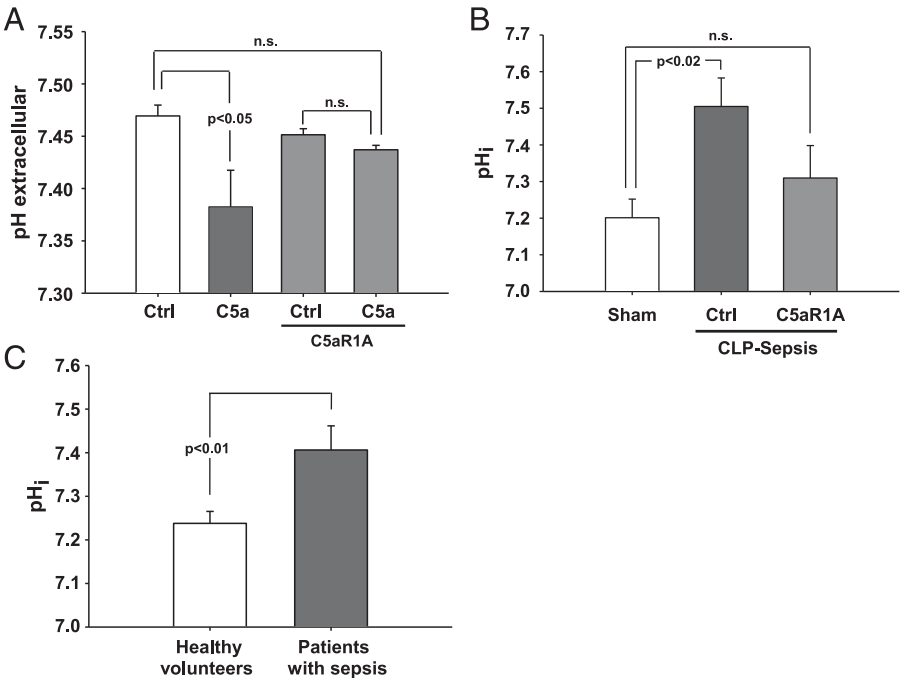

FIGURE 5. C5a-stimulated neutrophils increase extracellular pH in vitro and pH_i during experimental and clinical sepsis. (**A**) Effect of C5a stimulation of human neutrophils (10 nM, 15 min) on the pH of the extracellular milieu (HBSS without Ca^{2+}/Mg^{2+} + 0.1% glucose) in the presence or absence of C5aR1A (10 μM, 10 min pretreatment) (n = 7). (**B**) Neutrophil pH_i in mice after CLP-induced sepsis; immediately after CLP, mice were injected i.v. with the selective C5aR1A AcF[OPdChaWR] (1 μg/g body weight) or with a scrambled control peptide (n = 10–13). (**C**) pH_i in neutrophils from healthy volunteers and from patients with septic shock (n = 10). Ctrl, control; n.s., not significant.

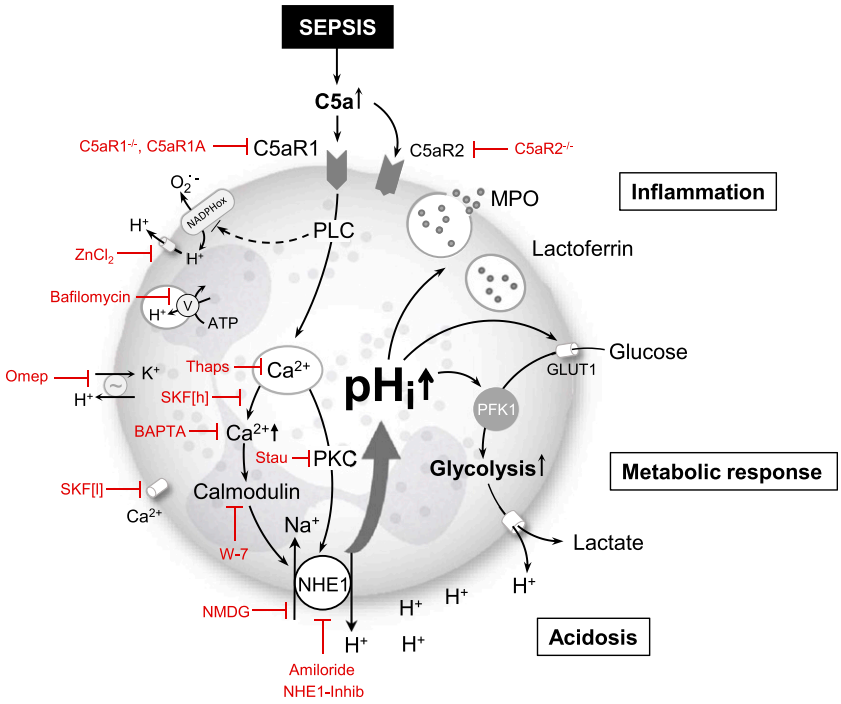

FIGURE 6. Model for the C5a-induced intracellular alkalinization of neutrophils. During sepsis, high amounts of C5a result in the activation of NHE1, which is dependent on upstream signaling of C5aR1, intracellular calcium, PKC, and calmodulin. The C5a-induced alkalinization leads to increased degranulation, glucose uptake, and activation of glycolytic flux, with subsequent release of lactate und protons. BAPTA, BAPTA-AM; NADPHox, NADPH oxidase; NMDG, *N*-methyl-D-glucamine; Omep, omeprazole; PLC, phospholipase C; SKF[h], SKF-96365 (50 μM); SKF[l], SKF-96365 (12 μM); Stau, staurosporine; Thaps, thapsigargin.

assumed for fMLF. Studies revealed that stimulation of neutrophils with fMLF resulted in an initial pH_i decrease through the accumulation of acidic metabolites that was counteracted by compensatory proton-induced NHE1 activation (10) and sustained intracellular alkalinization.

The increase in pH caused by C5a appears to be a prerequisite for the activation of neutrophil functions such as migration, chemotaxis, and superoxide production (8, 9). Subsequent amplification of reactive oxidants by MPO-dependent mechanisms, together with the microbicidal activity of lactoferrin, ensure the fundamental defense mechanisms of neutrophils. However, during sepsis, massive C5a generation results in excessive neutrophil activation and degranulation (3, 5, 38), which has the potential to trigger "innocent bystander" injury that promotes tissue damage and organ dysfunction (39, 40). The extracellular acidification by C5a may also contribute to this cytotoxic behavior. Importantly, inhibition of the C5a–C5aR1 interaction displayed protective effects at a cellular and organ level and improved the overall outcome (26). The present results suggest that the balancing effect on the pH_i homeostasis of neutrophils contributes to this protective behavior. In support of this, models of ischemia and reperfusion injury, known to be complement driven, also revealed protective effects on ischemia and reperfusion–related tissue necrosis via blockade of NHE1 (41, 42).

Beyond the functional activation, our study reveals that C5a-induced intracellular alkalinization led to enhanced activation of glycolytic flux, most likely via highly pH-sensitive phosphofructokinase-1 (PFK1). Mechanistically, PFK1 serves as a key control point within the glycolysis cascade and can be already fully activated by equivalent pH increases (10, 43) that can be achieved by C5a concentrations measured in patients with sepsis (31, 32). Moreover, the C5a–C5aR1 interaction induced a rapid pH-dependent increase in the glucose uptake, providing the source for the enhanced glycolytic flux that is basically required for activation of neutrophil functions. Thus, our present results are in line with the notion of C5a acting as insulin for neutrophils (44) and revealed a novel role for the complement-activation product as a molecular switch for metabolic activation of neutrophils. For monocytes, it has recently been shown that a mammalian target of rapamycin (mTOR)/hypoxia-inducible factor-1α (HIF-1α)–mediated metabolic shift toward aerobic glycolysis is fundamentally required for trained immunity (45). Although a specific immunological memory through DNA accessibility and gene expression is unlikely in neutrophils, a C5a-induced priming effect (46) via glycolysis is conceivable. In this context, leukocytes from septic patients exhibited an enhanced mTOR/HIF-1α–dependent expression profile of glycolytic genes during the acute phase, whereas in immune paralyzed cells, both glycolytic pathways and oxidative-metabolism pathways were substantially downregulated (47). However, the present C5a-induced activation mechanism of the glycolytic flux was independent of mTOR, because pretreatment of neutrophils with the mTOR inhibitor rapamycin did not impair the C5a effect (data not shown). Thus, the rapid metabolic-activation process by C5a seems to be independent of the known mTOR/HIF-1α axis and, instead, is mediated by a pH_i switch.

In patients with severe sepsis, enhanced lactate levels were identified as an independent predictor of sepsis mortality (48). Furthermore, systemic acidic conditions are frequently observed. In accordance with this, our previous animal studies revealed that, in rodent CLP sepsis, lactate levels were significantly increased, and an increased blood pH was associated with a poor outcome. All of these effects could be improved by a therapeutic C5a blockade (38, 49), suggesting that hyperlactatemia and extracellular acidification during sepsis might result from tissue hypoperfusion or microcirculatory disorders, as well as from complement-activated and primed immune cells. Mechanistically, hyperlactatemia and acidification of the extracellular milieu could be induced by increased glycolysis, with subsequent extrusion of lactate and protons and activation of the Na^+/H^+ exchanger due to the C5a-mediated increase in pH_i. This is of specific clinical relevance because our in vivo data confirmed a sepsis-induced increase in pH_i in neutrophils after CLP-induced sepsis, as well as during human sepsis. In rodent sepsis, this intracellular alkalinization could be completely abolished by C5aR1 inhibition. Therefore, complement-inhibitory strategies (50) might address immune surveillance (51) and coagulation cross-talk (52, 53)

during systemic inflammatory conditions, as well as balance basic physiological mechanisms critical for pH homeostasis and metabolic activity of neutrophils.

Acknowledgments

We thank Sonja Braumüller, Sonja Märsmann, and Barbara Reutter for outstanding laboratory assistance and Prof. Gunnar Ronquist for excellent advice regarding cell metabolism.

Disclosures

The authors have no financial conflicts of interest.

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